Acta vet. scand. 1972, *13*, 449—461.

From the Department of Medicine I, Royal Veterinary College, Stockholm, Sweden.

IMMUNOELECTROPHORETIC AND IMMUNODIFFUSION STUDIES ON THE SEMINAL PLASMA PROTEINS IN BOARS

By

Kjell Martinsson and *Kerstin Thorén*

MARTINSSON, KJELL and KERSTIN THORÉN: *Immunoelectrophoretic and immunodiffusion studies on the seminal plasma proteins in boars.* Acta vet. scand. 1972, *13*, 449—461. — The proteins of boar seminal plasma have been studied with immunoelectrophoresis and immunodiffusion tests. Four different proteins could be identified with immunoelectrophoresis of which all seemed to be specific for seminal plasma. One of these proteins with an electrophoretic mobility corresponding to gammaglobulin had no identity with either IgG or fibrinogen.

With immunodiffusion tests spur of serum proteins could be detected (albumin, transferrin and IgG).

The spontaneous precipitation of boar seminal plasma in agar gels could not wholly be avoided with alterations of pH and ionic strength in the agar gel or with absorption of seminal plasma with swine red blood cells.

One factor in seminal plasma which probably is of importance for the ability to agglutinate red blood cells was isolated and identified as a protein with an electrophoretic mobility corresponding to alphaglobulin. This protein could not be detected in blood serum.

seminal plasma; swine.

Characterization of the seminal plasma proteins of boars has hitherto only been performed with use of agar gel electrophoresis (*Bennet* 1965) and gel filtration (*Boursnell et al.* 1966). Four different fractions in the albumin-gammaregion were identified. Two of the fractions, in the beta- and gammaregion respectively, contained almost all of the total proteins and in the other two fractions only minute amounts of proteins could be detected. The use of gel filtration with Sephadex G 200 has revealed two distinct fractions (*Boursnell et al.* 1966), but no electrophoretic characterization of the fractions was performed.

The ability of boar seminal plasma to precipitate spontaneously has greatly complicated the separation by electrophoretic methods. Thus, *Bennet* has shown that boar seminal plasma only could be separated by agar gel electrophoresis at pH 7.0, ionic strength 0.3. Because of the high ionic strength the voltage was reduced to 8.4 V/cm and the electrophoretic run protracted to 75 min. In this way the precipitation of proteins in agar gel could be avoided.

Nelson & Boursnell (1966) have shown that the protein-precipitating activity was due to small amounts of basic proteins in the seminal plasma. Furthermore the precipitating activity could be removed by absorption with pig red blood cells, and it was suggested that the precipitating and red cell agglutinating activity was due to the same proteins.

The precipitating activity of boar seminal plasma is not a unique property among animals. Thus, seminal plasma of bulls, rabbits and rams also precipitates under certain conditions, but at an ionic strength of 0.1 and pH of 8.0 the precipitation cannot occur, and therefore electrophoretic separations can be performed without special arrangements (*Bennet*). It is obvious that two possibilities exist to obtain an electrophoretic separation of boar seminal plasma proteins judging from previous investigations: a) the use of a high ionic strength in the gel and a protracted electrophoretic run, b) an absorption of the seminal plasma with pig red blood corpuscles before the electrophoretic run to remove the precipitating activity.

The aim of this investigation is to separate the proteins in boar seminal plasma with immunoelectrophoretic and immunodiffusion methods and to identify the proteins with different specific antisera. Furthermore different methods will be used in order to avoid spontaneous precipitation thus facilitating the electrophoretic run.

MATERIAL AND METHODS

Seminal plasma. Semen was collected in an artificial vagina, filtered and centrifuged (5000 r.p.m. for 12 min.). Seminal plasma was used immediately or frozen at —20°C until use.

Antisera. Anti-seminal-plasma serum was obtained by immunizing a rabbit with 1 ml of seminal plasma suspended in an equal volume of Freund complete adjuvant, and 100 i.u. of ben-

zyl-penicillium and 50 ng of streptomycin were added. Three intramuscular injections were performed at intervals of one week. Then the same amount of antigen without adjuvant was injected three times with an interval of four days, and six days after the last injection 30 ml of blood was drawn and used as anti-seminal-plasma serum.

Anti-swine-serum and anti-swine-IgG were prepared according to the methods of *Martinsson* (1970).

Anti-swine-IgM, -IgG, -IgA, -transferrin and -albumin were prepared according to the methods of *Martinsson et al.* (a).

Absorption of seminal plasma with pig red blood corpuscles. Ten ml of heparinized blood was drawn from a grown pig, and the red blood corpuscles RBC) were washed five times with buffered saline, and a 20 % solution of the RBC was prepared. Then 1 ml of the RBC-solution was incubated for 30 min. at room temperature with 4 ml of the seminal plasma. A heavy agglutinate was formed and centrifuged off (3000 r.p.m. for 5 min.). This absorption was repeated (about eight times) until no or only a slight agglutinate was formed and then the supernatant was concentrated by means of polyethylene glycol to the original volume of the seminal plasma (4 ml).

Hemolyzing of the RBC-agglutinate and normal pig RBC. The agglutinate formed after absorption of seminal plasma was washed 10 times with buffered saline and then hemolyzed by adding half a volume of distilled water. Normal pig RBC was washed five times with buffered saline, and the packed RBC was then hemolyzed in the same way as the RBC-agglutinate.

Dialysis of seminal plasma. Five ml of native seminal plasma was dialyzed for 24 hrs. against veronal buffer pH 8.6; I 0.1. The precipitate formed was centrifuged off and washed five times with the same buffer and dissolved in 5 ml of 0.3 M-NaCl.

Immunoelectrophoresis. This was performed according to the method of *Scheideggar* (1955). Two different buffers were used in the agar:

1) veronal buffer pH 8.6; I 0.1.
2) phosphate buffer pH 7.0; I 0.3.

Immunodiffusion. This test was performed in agar of veronal buffer pH 8.6; I 0.1.

RESULTS

The immunoelectrophoretic analyses of native seminal plasma and swine serum against anti-seminal-plasma serum and anti-swine serum are seen in Figs. 1 and 2. It will be seen from Fig. 1 that four precipitates can be recognized, of which one is in the gamma-region, one in the beta-region and two in the beta- alpha-region. Only one or two very weak precipitates in the alpha-region are observed in immunoelectrophoretic analyses of swine serum against anti-seminal plasma (Fig. 1).

In Fig. 2 it will be seen that no distinct precipitate is visible after immunoelectrophoretic analyses of seminal plasma against anti-swine serum. From both Figs. 1 and 2 it is obvious that some of the proteins in seminal plasma have precipitated around the well in the agar (veronal buffer pH 8.6; I 0.1).

The immunoelectrophoretic separation of seminal plasma after agglutination with swine red blood corpuscles (RBC-treated plasma) and swine serum against anti-seminal plasma is seen in Fig. 3. The same precipitates can be recognized as in Fig. 1. A less precipitation of proteins around the well can be seen in Fig. 3 in comparison with Fig. 1.

The immunoelectrophoretic separation of RBC-treated plasma and swine serum against anti-swine serum is seen in Fig. 4. The same picture will be seen as in Fig. 2 with the exception of a slight precipitate in the albumin region between RBC-plasma and anti-swine serum. Also in this figure a less precipitate is visible around the well with the RBC-treated plasma in comparison with native plasma (Fig. 2).

The separation of native seminal plasma in agar of a higher ionic strength (veronal buffer pH 7.0; I 0.3) against anti-seminal plasma is seen in Fig. 5. No increased degree of separation can be observed in comparison with separation at a lower ionic strength (Fig. 1), but the precipitation around the well is decreased.

In Fig. 6 the separation of native seminal plasma and of the supernatant after dialysis against the buffer of the agar is seen (veronal buffer pH 8.6; I 0.1). No differences of the immunoelectrophoretic pattern can be observed. The precipitate obtained after the dialysis was washed with the same buffer and dissolved in 0.3 M-NaCl and then subjected to immunoelectrophoretic separation (Fig. 7). Judging from the immunoelectrophoretic pat-

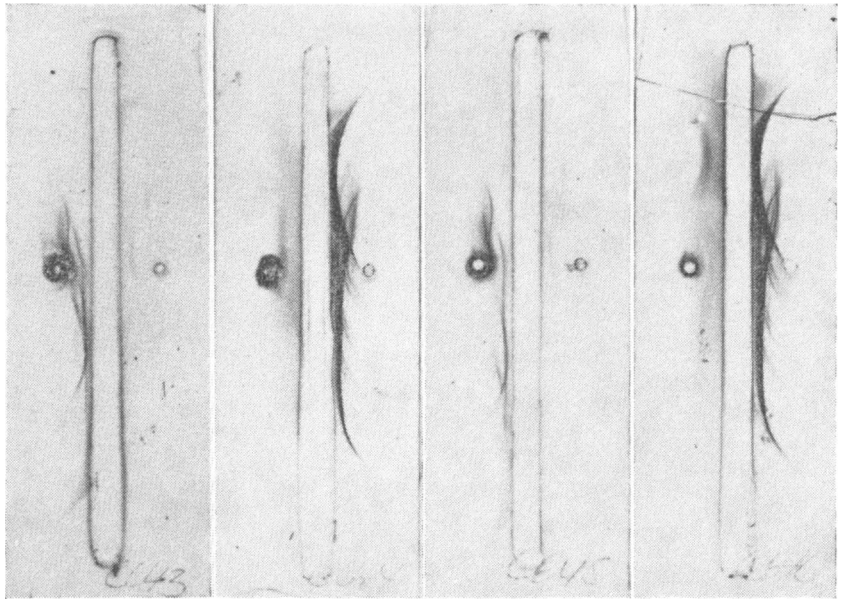

Figure 1. Immunoelectrophoresis of native seminal plasma (to the left) and swine serum (to the right) against anti-seminal-plasma serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar. Note the precipitation around the well.

Figure 2. Immunoelectrophoresis of native seminal plasma (to the left) and swine serum (to the right) against anti-swine serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar. Note the precipitation around the well.

Figure 3. Immunoelectrophoresis of RBC-treated seminal plasma (to the left) and swine serum (to the right) against anti-seminal-plasma serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar.

Figure 4. Immunoelectrophoresis of RBC-treated seminal plasma (to the left) and swine serum (to the right) against anti-swine serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar.

tern the dissolved precipitate contains the major proteins present in the seminal plasma.

The immunoelectrophoretic separation of swine blood plasma and RBC-treated seminal plasma against anti-seminal plasma is seen in Fig. 8. No precipitate in blood plasma (e.g. fibrinogen) corresponds to the precipitate in the gamma-region of the RBC-plasma.

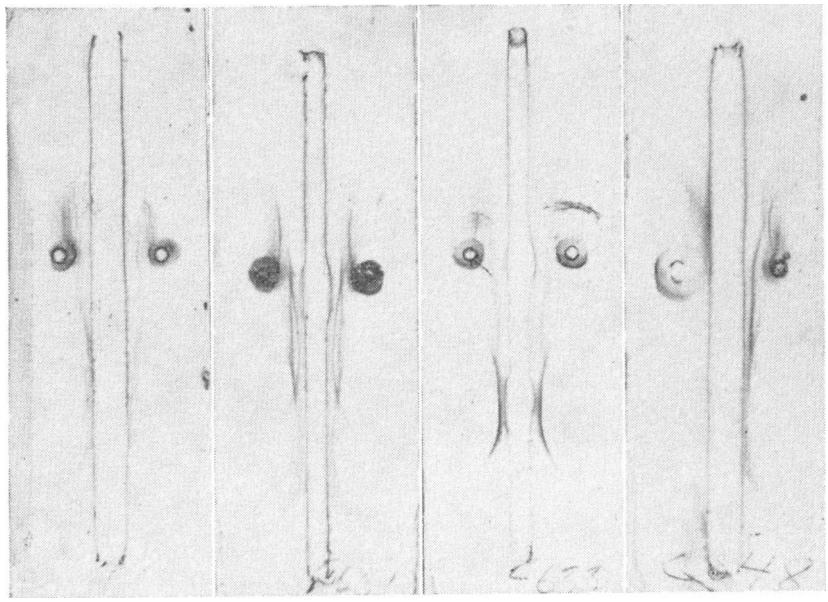

Figure 5. Immunoelectrophoresis of native seminal plasma (to the left and right) against anti-seminal-plasma serum. Phosphate buffer pH 7.0 and I 0.3 was used in the agar.

Figure 6. Immunoelectrophoresis of native seminal plasma (to the left) and of the supernatant after dialysis of seminal plasma against the buffer of the agar (to the right) against anti-seminal-plasma serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar.

Figure 7. Immunoelectrophoresis of the precipitate, dissolved in 0.3 M-NaCl, obtained after dialysis of seminal plasma as described in Fig. 6 (to the left and right) against anti-seminal-plasma serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar.

Figure 8. Immunoelectrophoresis of swine blood plasma (to the left) and RBC-treated seminal plasma (to the right) against anti-seminal-plasma serum. Veronal buffer pH 8.6 and I 0.1 was used in the agar.

The immunoelectrophoretic separation of swine serum and RBC-treated seminal plasma against anti-swine albumin, anti-swine transferrin and anti-swine IgG is seen in Figs. 9 a—c. It will be seen that no visible precipitate is obtained with the specific antisera and RBC-plasma.

The immunodiffusion tests of RBC-treated seminal plasma against anti-albumin, anti-transferrin, anti-IgA, anti-IgM and

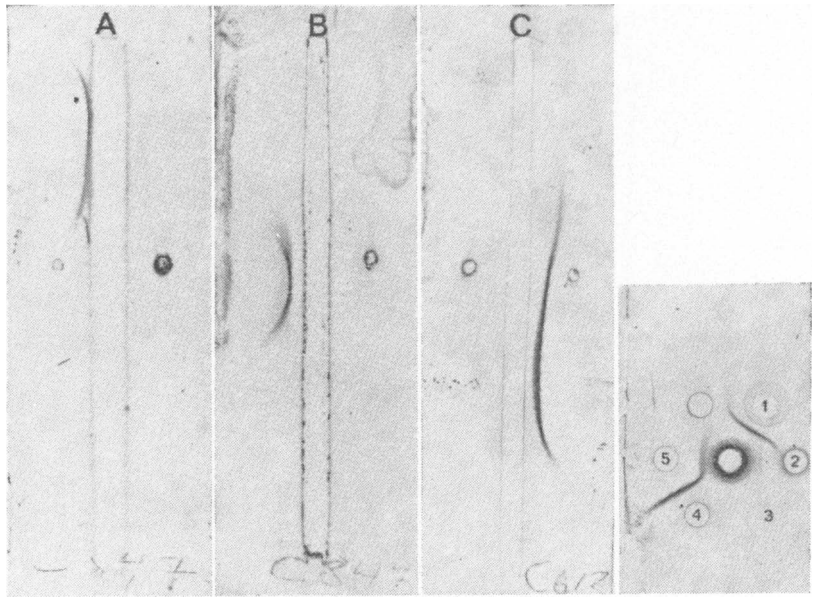

Figure 9 A. Immunoeletrophoresis of swine serum (left) and RBC-treated seminal plasma (right) against anti-swine-albumin serum.

Figure 9 B. Swine serum (to the left) and RBC-treated seminal plasma (to the right) against anti-swine-transferrin serum.

Figure 9 C. RBC-treated plasma (to the left) and swine serum (to the right) against anti-swine-IgG serum.

Figure 10. Immunodiffusion tests of RBC-treated seminal plasma (in the center) against 1) anti-swine albumin, 2) anti-swine transferrin, 3) anti-swine IgA, 4) anti-swine IgM and 5) anti-swine IgG. The reaction between 4) and 5) is due to an absorption of anti-swine IgM with swine IgG to get a monospecific anti-swine-IgM serum.

anti-IgG are seen in Fig. 10. A precipitate is visible between RBC-plasma and anti-albumin, anti-transferrin and anti-IgG.

The heavy agglutinate of swine red blood corpuscles formed by native seminal plasma was washed and hemolyzed and subjected to immunoelectrophoresis and immunodiffusion tests (Figs. 11—12). In the immunodiffusion tests (Fig. 12) a precipitate is visible between hemolyzed RBC-agglutinate and anti-seminal plasma but not between normal RBC which are hemolyzed and anti-seminal plasma. The precipitate does not correspond to either albumin, transferrin, IgG, IgA or IgM and seems

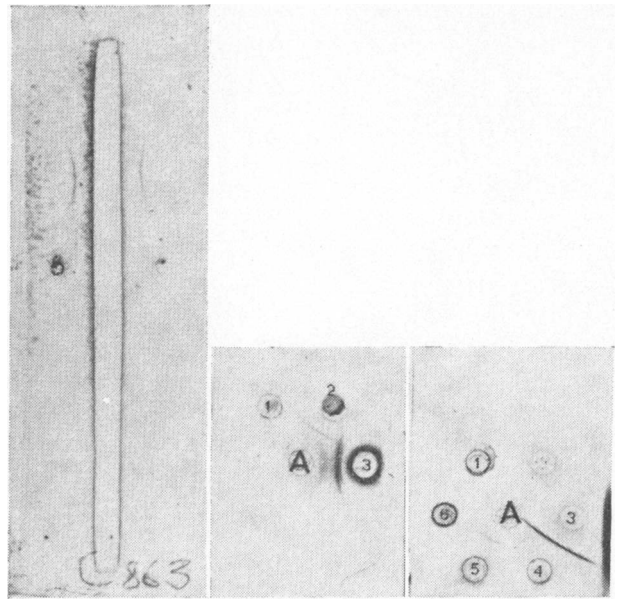

Figure 11. Immunoelectrophoresis of the hemolyzed agglutinate formed by pig RBC with native seminal plasma (to the left and right) against anti-seminal-plasma.

Figure 12. Immunodiffusion tests of A (anti-seminal plasma) against 1) normal hemolyzed pig RBC, 2) RBC-agglutinate formed by native seminal plasma and hemolyzed, and 3) native seminal plasma.

Figure 13. Immunodiffusion test of A (RBC-agglutinate formed by native seminal plasma and hemolyzed) against 1) anti-swine IgG, 2) anti-swine IgM, 3) anti-swine serum and 4) anti-seminal-plasma serum.

not to occur in blood serum (Figs. 11 and 13). After immunoelectrophoretic analysis of the hemolyzed RBC-agglutinate against anti-seminal plasma a precipitate is observed in the alpha-albumin region (Fig. 11).

DISCUSSION

In this investigation it has been shown that the proteins of swine seminal plasma can be separated by means of immunoelectrophoretic techniques. However, the spontaneous precipitation of the seminal plasma in the agar could not wholly be avoided by any of the three methods employed for the immunoelectro-

phoretic separation. By using an agar gel in a buffer of pH 8.6 and I 0.1 a relatively large precipitate arose around the well, but in spite of this four precipitates could be observed by using anti-seminal plasma as antiserum. This is in accordance with investigations performed by *Bennet* (1965) who found that the precipitation of boar seminal plasma occurred at pH 5.5—9.2 and at an ionic strength less than 0.15. Because of this finding, immunoelectrophoresis was performed at pH 7.0 and at an ionic strength of 0.3 (Fig. 5). The use of this ionic strength required protracted electrophoretic runs at low voltage to avoid heating (8.4 V/cm, 75 min. duration). The results indicate that no better separation is obtained by the use of a buffer of high ionic strength (Fig. 5) in comparison with a buffer with an ionic strength of 0.1 (Fig. 1). However, it is obvious that the degree of precipitation around the well is less by using a buffer of an ionic strength of 0.3, indicating that the spontaneous precipitation is decreased.

The finding that the spontaneous precipitation is not wholly avoided by using agar with a high ionic strength may partly be due to factors in the agar which have been shown to be able to precipitate even serum proteins (*Backemüller & Oerter* 1956, *Wierne* 1959).

As has been shown by *Nelson & Boursnell* (1966) the protein-precipitating and hemagglutinating properties of boar seminal plasma are largely attributable to the presence of minute quantities of basic proteins in boar seminal plasma. Therefore seminal plasma was allowed to agglutinate swine RBC repeatedly until no visible agglutinate was formed, and after centrifugation the supernatant was tested by immunoelectrophoresis (Fig. 3). In this case the same precipitates could be recognized as after immunoelectrophoresis of native seminal plasma (Fig. 1). However, a slight precipitation of proteins around the well can be seen. It indicates that it is not sufficient to remove the RBC-agglutinating activity of the seminal plasma in order to wholly exclude the spontaneous precipitation in the agar. As has been pointed out previously this finding may be due to factors in agar which are capable of precipitating proteins or to the fact that the precipitating and hemagglutinating properties of seminal plasma may be not entirely attributable to the presence of small quantities of basic proteins (*Nelson & Boursnell*).

No distinct precipitates can be seen after immunoelectrophoretic separation of seminal plasma against anti-swine serum

(Fig 2). It may indicate that most of the proteins in seminal plasma are specific for seminal plasma and do not occur in serum. Furthermore the immunoelectrophoretic separation of swine serum against anti-seminal plasma only gives rise to one or two very weak precipitates in the alpha-region (Fig. 1).

In order to support the suggestions mentioned above that most proteins in seminal plasma are specific for the seminal plasma, immunoelectrophoretic separations of seminal plasma have been run against monospecific anti-albumin-, anti-transferrin-, anti-IgM- and anti-IgG-serum (Figs. 9 a—c). It will be seen that no precipitate is obtained with any of the antisera.

However, it should be pointed out that the sensitivity of immunoelectrophoretic analyses is not high enough to detect minute quantities of proteins. Therefore immunodiffusion tests were performed of seminal plasma against the same monospecific antisera and anti-IgA serum in order to detect some of these serum proteins in seminal plasma (Fig. 10).

By this test albumin, transferrin and IgG could be recognized. It indicates that these proteins are only present in minute amounts as they are not detected by immunoelectrophoretic analyses.

Of special interest is the precipitate in the gamma-region obtained after immunoelectrophoresis of seminal plasma against anti-seminal plasma. From the results above it is obvious that this precipitate is not identical with gammaglobulin (IgG). To exclude the possibility that this precipitate corresponds to fibrinogen, swine blood plasma and seminal plasma was run against anti-seminal serum (Fig. 9). No precipitate was formed, and therefore it can be concluded that the precipitate in the gamma-region is not identical with fibrinogen, but must be a protein specific for seminal plasma as most of the other precipitates obtained after separation of seminal plasma. This finding is of great importance insofar that the protein in the gamma-region incorrectly may be noticed as gammaglobulin (IgG) by the use of gel- or paper-electrophoresis.

Boursnell et al. (1962) have shown the seminal vesicles to be the origin of most seminal plasma proteins of the boar, but it has not been shown whether only proteins specific of seminal plasma are synthetized in the seminal vesicles. In previous investigations (*Martinsson et al.* b) it was found that labelled albumin and IgG only in trace amounts were transferred from serum to seminal plasma. It may indicate that albumin and IgG detected

by immunodiffusion of seminal plasma in fact are derived from blood plasma.

The failure of detecting serum proteins such as IgM and IgA in seminal plasma may either be due to the low concentration of these proteins in serum or a less pronounced transmission from blood serum to seminal plasma.

The results of the present investigation indicate that the major proportion of the proteins of seminal plasma is specific for seminal plasma. Therefore these proteins only are detected by using an antiserum to seminal plasma, but it will not be possible to identify and characterize these proteins in detail until they are available in a pure state as has been shown in humans (*Klopstock et al.* 1963).

The specific properties of boar seminal plasma to precipitate in especially dilute solutions have been shown to be associated with the ability to agglutinate RBC (*Nelson & Boursnell*). In the present investigation it was shown that one protein could be isolated from RBC agglutinated by seminal plasma, and then hemolyzed and subjected to immunoelectrophoresis (Fig. 11). The protein has an electrophoretic mobility corresponding to albumin-alphaglobulin. By immunodiffusion tests (Figs. 12 and 13) it is shown that the protein does not occur in blood serum or in the supernatant of normal RBC which are hemolyzed. Therefore it may be concluded that the isolated protein is specific for seminal plasma and is perhaps responsible for the hemagglutinating properties of seminal plasma.

The protein component cannot be recognized after immunoelectrophoresis of seminal plasma against anti-seminal-plasma serum. This may be due to a low concentration of the protein in seminal plasma. Another possibility is that the protein is coupled to other proteins resulting in a precipitation, which was visible around the well in the agar. This assumption is supported by the fact that when the precipitate, obtained after dialysis of seminal plasma against a buffer of low ionic strength, is dissolved and subjected to immunoelectrophoresis, a precipitation is still obtained around the well (Fig. 7).

The precipitate obtained after dialysis of seminal plasma against buffers with an ionic strength of 0.1 seems to contain the major protein components of the seminal plasma (Fig. 7). Furthermore, no specific protein seems to have disappeared from seminal plasma after removal of the precipitate (Fig. 6). There-

fore it may be concluded that the precipitation of seminal plasma affects all of its protein components, and is probably caused by a single protein in a low concentration identified by immunoelectrophoresis and immunodiffusion and with a mobility corresponding to albumin-alphaglobulin.

REFERENCES

Bennet, J. P.: Quantitative comparisons of the proteins of the seminal plasmas of bull, ram, rabbit and boar by agar gel electrophoresis. J. Reprod. Fertil. 1965, *9*, 217—231.

Backemüller, W. & A. Oerter: Zonelectrophorese in agar gel. Arb. Paul-Ehrlich-Inst. 1956, *52*, 60.

Boursnell, J. C., P. Johnsson & J. Zamora: An electrophoretic and ultracentrifugal study of boar seminal plasma. Biochim. biophys. Acta 1962, *63*, 374—379.

Boursnell, J. C., M. Nelson & D. M. Cole: Studies on boar seminal plasma proteins. III. Fractionation by gel filtration, ion exchange and other means. Biochim. biophys. Acta 1966, *117*, 134—143.

Klopstock, A., R. Haas & A. Rimon: Immunoelectrophoretic analysis of seminal plasma. Fertil. and Steril. 1963, *14*, 530—539.

Martinsson, K.: Immunoglobulin therapy in piglets. Acta vet. scand. 1970, *11*, Suppl. 29.

Martinsson, K., K. Thorén, N. Holmgren & Å. Jönsson: To be published, a.

Martinsson, K., B. Gustafsson & G. Einarsson: Studies on the transmission of ^{125}I IgG and ^{125}I albumin from serum to seminal plasma in boars. To be published, b.

Nelson, M. & J. C. Boursnell: Studies on boar seminal plasma proteins. IV. Isolation of factors with hemagglutinating and protein-precipitating activity. Biochim. biophys. Acta 1966, *117*, 144—156.

Scheideggar, J. J.: Une micro-métode de l'immuno-électrophorèse. (A micro-method of immunoelectrophoresis). Int. Arch. Allergy 1955, *7*, 103—110.

Wierne, R. J.: Studies of agar gel electrophoresis. Arscia Uitgaven N.V., Brussels 1959, p. 180.

SAMMANFATTNING

Undersökningar medelst immunoelektrofores och immunodiffusion av proteiner i spermieplasma från galt.

Medelst immunoelektrofores och immunodiffusion har proteinkomponenterna i svinspermieplasman undersökts. Fyra olika distinkta proteiner kunde påvisas med immunoelektrofores, varav samtliga synes vara specifika för spermieplasma. Ett av proteinerna med en mobilitet motsvarande serum-gammaglobulin hade ingen identitet med varken IgG eller fibrinogen.

Medelst immunodiffusion kunde dessutom spår av serumproteiner påvisas ex. albumin, transferrin och IgG.

Förmågan hos svinspermieplasma att spontant precipitera kunde ej helt undvikas varken med ändring av pH och jonstyrka i agargelen eller med absorbering av röda blodkroppar från svin.

En faktor i spermieplasma som troligen är orsaken till dess förmåga att agglutinera röda blodkroppar har isolerats och visades vara ett protein med en mobilitet motsvarande alphaglobulin. Denna faktor kunde ej påvisas i blodserum.

(Received September 6, 1971).

Reprints may be requested from: Kjell Martinsson, Department of Medicine I, Royal Veterinary College, S-104 05 Stockholm 50, Sweden.